AD_____

Award Number: DAMD17-98-1-8162

TITLE: Tropomyosin-1: A Putative Tumor Suppressor and a Biomarker of Human Breast Cancer

PRINCIPAL INVESTIGATOR: Gaddamanugu Prasad, Ph.D.

CONTRACTING ORGANIZATION: Wake Forest University School of Medicine
Winston Salem, North Carolina 27157

REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual Summary (27 Jul 99 - 26 Jul 00)

4. TITLE AND SUBTITLE
Tropomyosin-1: A Putative Tumor Suppressor and a Biomarker of Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8162

6. AUTHOR(S)
Gaddamanugu Prasad, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wake Forest University School of Medicine, Winston Salem, North Carolina 27157
E-MAIL:
gprasad@wfubmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Changes in the expression of microfilament-associated proteins such as tropomyosins, are associated with the transformed phenotype. Our previous work demonstrated 1. a complete loss of expression of tropomyosin-1 (TM1) in human breast carcinoma cell lines, indicating that it is a pivotal early event during the neoplastic transformation of mammary epithelium, and; 2. TM1 is a suppressor of the malignant phenotype. In this work we have tested the hypothesis that TM1 is putative biomarker and tumor suppressor of breast cancer. To facilitate the analysis of tumor specimens, we developed novel TM1-specific antibodies. These antibodies are currently being tested in immunohistochemical screening of breast tumors. Restoration of TM1 expression in MCF-7 (MCF-7/T) cells resulted in slower growth rate and expression of markers associated with normal differentiation status. MCF-7/T cells remain sensitive to growth control by estrogen, and TM1 re-expression appears to alter the interaction of E-cadherin-catenin complex with microfilaments. More significantly, MCF-7/T cells failed to grow under anchorage-independent conditions. Thus, TM1 appears to be essential for normal growth and differentiation of mammary epithelium. Abolition of TM1 expression appears to be necessary for malignant transformation by multiple oncogenic modalities. Together, our findings demonstrate that TM1 is a class II tumor suppressor.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer; tropomyosin; cytoskeleton; marker; tumor suppressor	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Section 4

Introduction

Title: Tropomyosin-1, A Putative Tumor Suppressor and a Biomarker of Breast Cancer.
Grant #: DAMD17-98-18162.

Changes in the expression of cytoskeletal proteins during the neoplastic transformation of cells are very well documented. While many such alterations are a consequence of the transformation *per se,* changes in the expression of some key proteins contribute to the malignant transformation. Earlier work from this laboratory identified that down regulation of Tropomyosin-1 (TM1), an actin-binding protein, is an important and common biochemical change in many human breast carcinoma cell lines. We also demonstrated that TM1 is a suppressor of malignant transformation induced by several oncogenes. The goal of this project is to evaluate the role of TM1 in breast cancer. Specifically, we proposed to assess the value of TM1 as a potential marker of breast cancer, and whether TM1 could suppress the transformed phenotype of breast carcinoma cells.

Section 5: Body

Annual Summary

1.TM1 expression in normal and malignant breast tissue: Normal epithelial cells express 7 different tropomyosin (TM) isoforms (1). In breast carcinoma cells severe disruptions in TM expression occur, as evidenced by the loss of multiple TMs. The complete loss of TM1 is consistently observed in a large number of cell lines tested. Suppression of other TM isoforms is also documented in breast carcinoma cells, but such changes are more variable. These data suggest that suppression of TM1 expression is common in all mammary neoplasms and we are assessing whether TM1 could be a marker of breast cancer.

We hypothesized that in the malignant breast epithelial cells TM1 expression would either be absent or significantly lower than their normal counterparts (2); the malignant cells, however, are expected to express at least some of the other 6 TMs. Since all TMs share a high degree of sequence homology, most of the available antisera do not permit accurate quantification of TM1 in the tissues by immunohistochemistry. To overcome this obstacle, we have developed a number of antipeptide antibodies that specifically recognize TM1. We have tested the utility of these reagents in immunoblotting using cell lines with known TM expression profiles. For example, normal MCF-10A cells express all TM isoforms, including TM1 and TM38 which co-migrate on SDS-polyacrylamide gels. In MCF-7 breast carcinoma cells, neither of these proteins are expressed, while MDA MB231 cells express TM38, but not TM1. When TM1-specific antibodies were used in immunoblotting, only MCF10A cells showed reactivity, attesting to the specificity of the reagents.

We have used one such antibody, TM20, for immunohistochemical analysis. In the initial experiments, we found that TM20 showed positive staining with normal mammary epithelium, while the malignant ducts lacked/reduced reactivity. This antibody is now being used for large scale screening experiments at the Wake Forest University School of Medicine.

In the event that we encounter any problems with TM20 reagent in screening, we will use other antibodies available generated in this laboratory or develop RNA–based detection methods such as in situ hybridization or Laser Capture Microscopy-RT-PCR methods (3, 4). This CDA enables the PI to learn and apply these techniques to his specific project.

2. Effects of expression in human breast carcinoma cell lines: Earlier work from this laboratory has demonstrated that TM1 is a suppressor of the malignant transformation and that it is a class II tumor suppressor(5, 2, 6, 3, 4). With a goal to investigate the causal role of TM1 in breast cancer and to whether novel breast cancer therapies based on TM1 would be possible, the following experiments were initiated. The well-studied MCF-7 cells were used as a model of breast cancer. MCF-7 cells do not express any detectable TM1. TM1 expression was restored in MCF-7 cells by retroviral mediated gene transfer and individual cell lines were generated. As indicated in the previous report, cell lines expressing TM1 are of revertant phenotype. This was evidenced by a decrease

in growth rate and the abolition of anchorage independent growth. Furthermore, the revertant cells remain under the growth controls of estrogen.

Investigations into the mechanism of TM1-mediated reversion suggested the involvement of E-cadherin-βcatenin signaling pathway. The total expression of e-cadherin or any of the catenins is altered in the revertants; instead, a reorganization of these adherens proteins was observed. The revertant cells displayed well organized cell-cell adhesion junctions. E-cadherin and β-catenin are better organized at the cell-cell boundaries and are more tightly linked to the cytoskeleton in the revertants than in the parental MCF-7 cells. A manuscript describing these results is in preparation and expected to be communicated soon.

Current efforts are focused on whether the altered expression of two key growth factors contributes to the revertant phenotype of MCF-7 cells expressing TM1. Expression of TGFα and amphiregulin will be assessed in the malignant and the revertant MCF-7 cells. In addition, we are extending the investigations on the β-catenin signaling pathways.

3. Induction of transformed phenotype by suppression of TM1 expression: In order to test whether the loss of TM1 expression could lead to the expression of malignant transformation of mammary epithelium, antisense suppression of TM1 is proposed. TM1 was subcloned in antisense direction in the retroviral vector pBNC and antisense packaging cells of PA317 are generated. At this point we are considering more effective and novel strategies for antisense suppression are considered. First, we are exploring whether ribozyme mediated TM1 suppression would be more effective than the 'classical' antisense experiments. Second alternative that is being considered is cre-lox mediated generation of TM1 knock out cell lines of normal mammary epithelial cells. The CDA award makes it possible to learn and apply these techniques to my research project.

4. Structure-function relationship of TM1-mediated tumor suppressive effects: Work on creating chimeras of TM1 (a tumor suppressor) and TM2 (not a tumor suppressor) has been initiated. We have completed the site directed mutagenesis to introduce a silent mutation to create a HindIII restriction site. This was accomplished by PCR and the resultant variants of TM1 and TM2, designated as 'TM1-h' and 'TM2-h' respectively, containing the HindIII site were sequenced. Switching of the carboxy (at AvaI) site and the central exons (HindIII-AvaI) is now in progress. At this point, we have chosen to test the use of epitope-tagged TMs in a different cell system. Depending on those results, we will either continue to work with the constructs that are generated, or employ the epitope-tagged constructs for easy monitoring in the transfection experiments.

Section 6

Key Research accomplishments

- Several TM1-specific antisera have been developed and their specificity is tested.
- Screening of breast tissues for TM1 expression is in progress.
- In addition to originally proposed immunohistochemical screening, we are now employing *in situ* hybridization and Laser Capture microscopy-RT PCR strategy.
- Restoration of TM1 expression reverts MCF-7 cells.
- The revertant MCF-7 cells display improved cell-cell adhesion complexes, with e-cadherin and β-catenin relocalized to the cell-cell junctions.

Section 7.

Reportable Outcomes

Manuscripts and Abstracts

1. Manuscripts:
   1.1. Restoration of tropomyosin-1 TM1 reverts the malignant phenotype of human breast cancer cells. Mahadev Kalyankar, Barbara Vonderhaar, David Solomon and G. L. Prasad. (in preparation)
   1.2. Tropmyosin-1 induced cytoskeletal reorganization involves Rho kinase signaling. Vanya Shah and G. L. Prasad (manuscript communicated for publication)
2. Two abstracts were presented:
   2.1. Mahadev Kalyankar, James G. Caya, Barbara K. Vonderhaar and **G. L. Prasad**. Tropomyosin-1 is a tumor suppressor and a marker of breast cancer. 32nd Annual meeting of American Society for Cell Biology, Washington DC, December 15th 1999.
   2.2. Tropomyosin-1 Is A Suppressor Of The Malignant Phenotype Of Breast Cancer. G. L. Prasad and Mahadev Kalyankar, Presented at the Era of Hope meeting at Atlanta. June 8-12, 2000.

Employment opportunities:

The P. I. has received several employment opportunities. He has taken up a position at the Wake Forest University Comprehensive Cancer Center in the Departments of General Surgery and Cancer Biology beginning January 1, 2000.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8162 supported this work.

Section 8

Conclusions

In summary, the major accomplishment of our work for this year is to demonstrate that TM1 indeed is a tumor suppressor of breast carcinoma cells. We showed that restoration of TM1 expression in MCF-7 model cell system, abolishes anchorage-independent growth, significantly decreases the growth rates, while not altering the estrogen growth controls. We also found that TM1 might mediate these effects through e-cadherin-βcatenin pathway.

These findings could have important implications in understanding biology of breast cancer and possibly exploring novel therapies. First of all, our studies indicate that the assembly of cell-cell adhesion junctions can be modulated by changes in microfilament associated proteins. This suggests that microfilament proteins through their ability to regulate the assembly of cadherin-catenin junctions could, in fact, alter the gene expression via TCF-LEF/-βcatenin pathway. This possibility sheds light on the TM1-mediated suppression of the revertant phenotype, which is being investigated.

A second implication is to test whether TM1 can be used for gene therapy of breast cancer. Since TM1 appears to be a transformation-specific suppressor, it is an attractive therapeutic target. We are planning to explore this possibility by employing adenoviral vectors for the gene delivery.

Section 9

References

1. Bhattacharya, B., Prasad, G. L., Valverius, E. M., Salomon, D. S. & Cooper, H. L. (1990) Tropomyosins of human mammary epithelial cells: consistent defects of expression in mammary carcinoma cell lines. Cancer Res. 50: 2105-2112.
2. Braverman, R. H., Cooper, H. L., Lee, H. S. & Prasad, G. L. (1996) Anti-oncogenic effects of tropomyosin: isoform specificity and importance of protein coding sequences. Oncogene. 13: 537-545.
3. Kuecker, S. J., Jin, L., Kulig, E., Oudaogo, G. L., Roche, P. C. & Llyod, R. V. (1999) Analysis of PRL, PRL-R, TGFb1, and TGFb-RII gene expression after Laser Capture Microdissection. Applied immunohitochemistry & Molecular Morphology. 7: 193-200.
4. Jin, L., Thompson, C. A., Qian, X., Kuecker, S. J., Kulig, E. & Lloyd, R. V. (1999) Analysis of anterior pituitary hormone mRNA expression in immunophenotypically characterized single cells after laser capture microdissection. Laboratory Investigation. 79: 511-512.
5. Prasad, G. L., Fuldner, R. A. & Cooper, H. L. (1993) Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene. Proc. Natl. Acad. Sci. USA. 90: 7039-7043.
6. Prasad, G. L., Masuelli, L., Raj, M. H. & Harindranath, N. (1999) Suppression of src-induced transformed phenotype by expression of tropomyosin-1. Oncogene. 18: 2027-2031.

Appendix

(Abstract presented at the Era of Hope meeting at Atlanta, June 2000)

TROPOMYOSIN-1 IS A SUPPRESSOR OF THE MALIGNANT PHENOTYPE OF BREAST CANCER

G. L. Prasad and **Mahadev Kalyankar,** Departments of Surgery and Cancer Biology, Wake Forest University Comprehensive Cancer Center, Winston-Salem, NC 27157.
e-mail: gprasad@wfubmc.edu

Changes in the expression of microfilament-associated proteins such as tropomyosins (TMs), are associated with the transformed phenotype. Our previous work demonstrated: 1. the loss of expression of tropomyosin-1 (TM1) is a common biochemical event found in many transformed cells, and; 2. TM1 is a suppressor of the malignant phenotype induced by retroviral oncogenes. While many defects in the expression of TMs are noted in human breast carcinoma cell lines, TM1 expression was consistently abolished in all the human breast carcinoma cell lines tested. In this work we have tested the hypothesis that TM1 is a tumor suppressor of breast cancer and its suppression is an early, pivotal event during the neoplastic transformation of mammary epithelium.

MCF-7 cells, which have lost the expression of TM1, were utilized as a model of human breast cancer. Restoration of TM1 expression in MCF-7 cells (MCF-7/T) resulted in slower growth rate and expression of markers associated with normal differentiation status. MCF-7/T cells remain sensitive to growth control by estrogen. More significantly, MCF-7/T cells failed to grow under anchorage-independent conditions. TM1 re-expression alters the interaction of E-cadherin-catenin complex with the cytoskeleton. Re-localization of E-cadherin and β-catenin, but not α- or γ-catenins, to cell-cell contacts was observed in the revertants, indicating that it could play a significant role in suppression of the malignant phenotype. Thus, TM1 appears to be essential for normal growth and differentiation of mammary epithelium. Together, our findings demonstrate that TM1 is a class II tumor suppressor.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8162 supported this work.